

In Vitro Effect of Lithium Chloride on Adipose Tissue Derived Stem Cells Proliferation and Growth Kinetic

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Received: 17 September 2025

Revised: 15 November 2025

Accepted: 05 December 2025

What's Known

- Mesenchymal stem cells (MSCs) are widely used and are powerful sources of cell-based transplantation therapies.
- There is always a need to apply safe and simple methods to increase the cell number and to prevent cell apoptosis.
- Lack of enough data in the literature on the effect of lithium chloride on safe proliferation of the MSCs and their protection from apoptosis to reach the maximum number of cells needed for cell therapy, especially for adipose tissue-derived MSCs.

What's New

- Addition of lithium chloride to cell culture media did not induce any abnormality in cell morphology.
- Lithium chloride had an increasing impact on the proliferation of adipose tissue-derived MSCs.
- Lithium chloride led to a decrease in *BAX* gene revealing its protective effect on apoptosis.

Abstract

Background: Adipose tissue-derived stem cells (AdSCs) are widely used for regenerative medicine purposes. There is always a need to accelerate cell proliferation and protect the cells from apoptosis when cell transplantation, especially AdSCs, is targeted. Therefore, this study evaluated the effect of lithium chloride on the proliferation and growth kinetic of AdSCs.

Methods: The study was undertaken in the Stem Cell Technology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran, in 2020. Adipose tissue specimens were provided from the abdominal region of a 35-year-old woman. In an *in vitro* study, human AdSCs were characterized morphologically, by osteo- and adipogenic differentiation properties, and by flow cytometry. They were later treated with lithium chloride to evaluate its effect on cell proliferation and the growth kinetics of AdSCs. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay investigated lithium chloride effect on the proliferation rate and apoptosis of AdSCs. Quantitative Real Time Polymerase Chain Reaction (qPCR) was carried out to determine the expression of apoptosis genes of Apoptosis Regulator BCL2 Associated X (*BAX*) and B-cell lymphoma 2 (*Bcl-2*) protein family.

Results: AdSCs showed mesenchymal characteristics, and lithium chloride at a dose of 6 μ M had an increasing impact on cell proliferation and protected the cells from apoptosis. A non-significant increase in expression of the *Bcl-2* gene ($P=0.057$) and a significant decrease in *BAX* gene ($P=0.034$) expression were visible.

Conclusion: Our findings indicate that the addition of lithium chloride to the culture medium enhanced the proliferation of AdSCs and protected them against apoptosis.

Please cite this article as: Homayoon M, Mehrabani D, Edalatmanesh MA, Shariati M, Amini M. *In Vitro* Effect of Lithium Chloride on Adipose Tissue Derived Stem Cells Proliferation and Growth Kinetic. Iran J Med Sci. 2026;51(6):445-454. doi: 10.30476/ijms.2026.108735.4372.

Keywords • Lithium chloride • Adipose tissue • Mesenchymal stem cells • Cell proliferation • Regenerative medicine

Introduction

In recent years, regenerative medicine has gained considerable attention by using cell therapy and tissue engineering, and has opened a new window in the treatment of several diseases.^{1, 2} Various stem cells from different tissue sources have been isolated and expanded for cell transplantation purposes, such as bone marrow,³ menstrual blood,⁴ dental pulp,⁵ and adipose tissue.⁶

Adipose tissue-derived stem cells (AdSCs) as an available source for cell therapy have been widely used in regenerative medicine.⁷ They can be easily differentiated into other cell lineages via appropriate induction conditions.^{3, 6, 7}

However, these cells usually do not have a long survival rate, and they may rapidly disappear if serum deprivation is prolonged, as apoptosis mechanisms can be initiated.⁸ The protective role of lithium chloride against oxidative stress caused by serum deprivation has been shown before,^{9, 10} and it can also increase stem cell proliferation.¹¹ Lithium chloride is the smallest alkali metal and an essential trace element with an atomic weight of 6.9 found in grains, vegetables, and supplemented in drinking water and is a mood-stabilizing drug widely used in the treatment of bipolar affective disorders.¹² The therapeutic effects of lithium in the treatment of central nervous system (CNS) injury and degenerative diseases such as Alzheimer's disease,¹³ Parkinson's disease,¹⁴ Huntington's disease,¹⁵ stroke,¹⁶ and spinal cord injuries¹⁷ have been demonstrated. During early development of the CNS, it can have dramatic effects on neurogenesis through direct inhibition of glycogen synthase kinase-3 β (GSK-3 β) and Wnt/b-catenin pathways.¹⁸

The *in vitro* effect of lithium chloride on cell proliferation and apoptosis of mammary epithelial cells has been described before.¹⁹ Lithium chloride (LiCl) can inhibit inflammation and apoptosis in mammary epithelial cells. It enhances the *KRT35* gene and activate Wnt/ β -catenin and mammalian target of rapamycin (mTOR) signaling pathways that can promote cell proliferation.¹⁹ It was shown that lithium can impact on dentinogenesis and osteogenesis in primary pulp cultures,²⁰ inhibit iron dysregulation and ferroptosis in induced pluripotent stem cells,²¹ prevent apoptosis in neural progenitor cell,²² and affect immunomodulation and differentiation of stem cells.²³ As mesenchymal stem cells (MSCs) are powerful tool in regeneration and repair of injured tissues, can differentiate into various tissues, have immunomodulatory and anti-inflammatory effects, self-renewal capacity, stemness maintenance, and plasticity, they can be a proper candidate for cell transplantation purposes.^{1-3, 6, 7} Therefore, based on lack of enough data in literature on the effect of lithium chloride on safe proliferation of the MSCs and their protection from apoptosis to reach the maximum number of cells needed for cell therapy especially for AdSCs, this study was undertaken to determine the *in vitro* effect of lithium chloride on proliferation, growth kinetic, and apoptosis of AdSCs.

Materials and Methods

The study protocol was approved by the Ethics Committee of Shiraz Islamic Azad University (IR.IAU.SHIRAZ.REC.1400.016). The study was undertaken in Stem Cell Technology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran in 2020.

Isolation of Adipose Tissue Stem Cells

Adipose tissue specimens were provided from the abdominal region of a 35-year-old woman who had been referred for abdominoplasty to Ghadir Mother and Child Hospital, affiliated to Shiraz University of Medical Sciences, Shiraz, Iran; while a written consent form was provided from the patient. The sample was rapidly transferred to the Cell Culture Laboratory of the Stem Cell Technology Research Center of Shiraz University of Medical Sciences, Shiraz, Iran in sterilized Falcon tube (BD, USA) containing phosphate buffer saline (PBS, Sigma, USA), 1% penicillin streptomycin (Sigma, USA), and amphotericin (Sigma, USA) for further investigation.⁶

The tissue samples were washed several times with phosphate-buffered saline (PBS, Gibco, USA) to remove the blood cells, and then were treated with 1.5 mL of 0.2% collagenase enzyme type 1 (Gibco, USA) for 40 min and placed in an incubator at 37 °C with 5% CO₂ and saturated humidity. Five mL of Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA) was added to the tube, and after passing through a 0.7 μ m filter, they were centrifuged for 5 min at 1200 revolutions per minute (rpm). Removal of the supernatant was undertaken, and then re-suspension of the remained cell pellet was carried out in 1 mL of DMEM supplemented with 1% penicillin-streptomycin and 10% FBS. The cell suspension was later transferred into a culture flask that already contained 4 mL of DMEM supplemented with 1% penicillin-streptomycin and 10% FBS. The culture flask was then placed in an incubator with 5% CO₂ and saturated humidity at 37 °C. The media change was planned every 3 days until reaching a cell confluence of 80%, which was determined under an inverted microscope (Nikon, Japan) equipped with a digital camera (Olympus, Japan) for taking the needed images. At a cell confluence of 80%, the cells were subcultured by subjecting them to 0.25% (w/v) trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, USA) till reaching (passage 3).⁶

Cell Characterization

For characterization of human AdSCs, the cells were evaluated morphologically, by *in vitro*

osteogenic and adipogenic inductions, and by flow-cytometry to assess for positive expression of mesenchymal (cluster of differentiation [CD73] and CD90) markers and negative expression of hematopoietic markers (CD34 and CD45). Morphological analysis showed that AdSCs up to passage 4 displayed fibroblast-like morphology when examined with an inverted microscope equipped with a digital imaging system.^{6,7}

To induce osteogenic differentiation, AdSCs were transferred in 6-well plates, while the osteogenic media consisted of the complete culture medium supplemented with 50 μ M ascorbic acid (Merck, Germany), 15% FBS, 10 mM glycerol 3-phosphate (Merck, Germany), and 100 nM dexamethasone (Sigma, USA). The cell expansion was followed for 3 weeks until reaching a cell confluence of 80%; while every 3 days, media change was done. Then, the cells were fixed in 10% neutral-buffered formalin for 20 min. Washing of fixed cells was carried out using deionized water for several times and were later stained with 1.4% Alizarin Red solution solved in deionized water (Sigma, USA) at pH of 4.1.^{6,7}

To induce adipogenic differentiation of AdSCs, the cells were seeded in 6-well plates consisted of the complete culture medium supplemented with adipogenic medium of 200 μ M of indomethacin (Sigma, USA), 100 nM dexamethasone, 15% FBS, and 100 μ M ascorbic acid for 3 weeks till reaching a cell confluence of 80%. Then, the cells were fixed in 10% neutral-buffered formalin (Merck, Germany) for 20 min. Thereafter, the cells were washed several times with deionized water at pH of 4.1 and stained with 0.5% Oil Red-O (Sigma, USA) solved in 2-propanol solution (Merck, Germany) for 2 hours.^{6,7}

3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

MTT assay was undertaken to investigate the impact of lithium chloride on cell proliferation; while AdSCs were seeded in 96-well plates (5,000 cells/200 μ L). Media change happened after 24 hours with either solution of (i) just culture media (control), or (ii) lithium chloride (Sigma-Aldrich, St. Louis, MO, USA). Lithium chloride was used at various concentrations (2, 4, 6, 8, 10, 20, 40, 70, 90, 200 μ M of lithium chloride) that was added to the culture media. After one day, 20 μ L of a solution consisting of 5 mg/mL of dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT: Sigma-Aldrich, USA) was added to each well. The culture wells were transferred to an incubator for 4 h with 5% CO₂ and saturated humidity at 37 °C. The media was

later removed, and the formazan crystals were dissolved by the addition of 200 μ L of DMSO/well (Merck, Germany).¹

Cell Viability

Evaluation of cell viability was undertaken using a microplate reader (Floustar Omega, BMG Lab Tech, Germany) (n=4) at optical density of 570 nm. The calculation was carried out using the formula:¹

$$\text{Survival rate}\% = \frac{A_{\text{sample}} - A_{\text{b}}}{A_{\text{c}} - A_{\text{b}}} \times 100$$

where Ac and Ab represent the absorbance in the control and blank wells, respectively.

Growth Kinetic

Growth kinetics was determined using a 0.4% trypan blue (Sigma, USA) exclusion test. AdSCs were cultured in 24-well plates (22,000 cells/well) and counted until 6 days under a phase contrast microscope (Olympus, FSX100, Japan) using a Neubauer hemocytometer slide, while the control cells were compared with the addition of 6 μ M of lithium chloride to the culture media. The population doubling time (PDT) was determined and the growth curve was plotted using the following formula, where *T* is the incubation time in hours, *X_b* is the cell number at the beginning of incubation, *X_e* is the cell number at the end of incubation, *ln* denotes the natural logarithm (log_e), *e* is Euler's number, *v* is the volume, and *n* is the frequency.²⁴

$$PDT = \frac{T \ln 2}{\ln\left(\frac{X_e}{X_b}\right)}$$

$$N = \frac{n_1 + n_2 + n_3 + n_4}{4} \times 2 \times v \times 100$$

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

To perform qPCR and to determine the Bax and Bcl-2 gene expressions, RNA extraction was carried out using an RNA extraction kit (Cinna Gen Inc., Tehran, Iran) from the harvested AdSCs 48 hours after exposure of the cells to lithium chloride. A Nanodrop™ spectrophotometer (Nanodrop; Thermo Fisher Scientific Inc., USA) was applied to evaluate the quantity and quality of recovered RNA and to assess the ratio of optical density (A260/A280 and A260/A230). Later, preparation of cDNA was done using 1,000 ng total RNA in a first-strand cDNA synthesis reaction applying a Revert Aid™ first-strand cDNA synthesis kit (Thermo Fisher Scientific Inc., USA).¹

To assess apoptosis in AdSCs treated with lithium chloride, apoptosis regulator BCL2 associated X (*BAX*) and B-cell lymphoma 2 (*Bcl-2*) protein family genes were selected as target genes and TATA box binding protein (*TBP*) as a housekeeper control gene. The gene sequences and primer sets were determined by applying the National Center for Biotechnology Information (NCBI) database employing primer3 software (table 1). The qPCR was conducted applying SYBR Green I as reporter dye and Step One Real Time PCR reactions (Applied Biosystems, USA). Totally, 200 nM of each primer was utilized to target the specific sequence in each reaction. The qPCR was set at 94 °C for 10 min, 40 cycles at 94 °C for 15 sec, and 40 cycles at 60 °C for 60 sec. The melting curve analysis ramped from 65 °C to 95 °C. The amplification signals were normalized to *TBP* cycle threshold (Ct). Two^{-ΔΔCt} method was used to compare the mRNA levels, which were presented as fold change in the analysis. Each reaction was

performed in triplicate to guarantee reproducibility and statistical reliability.¹

Statistical Analysis

Data were statistically analyzed by Prism software (version 6.0, Graph Pad Software Inc., USA) using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for comparisons. A repeated-measures ANOVA was applied to the dataset, considering time as the repeated variable. A P value less than 0.05 was statistically considered significant. All results are expressed as the mean±standard deviation (SD).

Results

Cell Characterization

AdSCs at different passages were demonstrated to be adherent to culture flasks and were spindle-shape in morphology (figure 1 A-D).

Table 1: The sequences of the defined primers

Genes	Primer sequence	Size (bp)
<i>Bax</i>	Forward: 5'- GCCCTTTGCTTCAGGGTTTCA -3'	108
	Reverse: 5'- CAGCTTCTTGGTGGACGCAT -3'	
<i>Bcl-2</i>	Forward: 5'- ACGAGTGGGATGCGGGAGATGTG-3'	245
	Reverse: 5'- GCGGTAGCGGCGGGAGAAGTC-3'	
<i>TBP</i>	Forward: 5'- GGATAAGAGAGCCACGAACCAC-3'	139
	Reverse: 5'- TTAGCTGAAAACCCAACTTCTG-3'	

bp: Base pair; TBP: TATA box binding protein

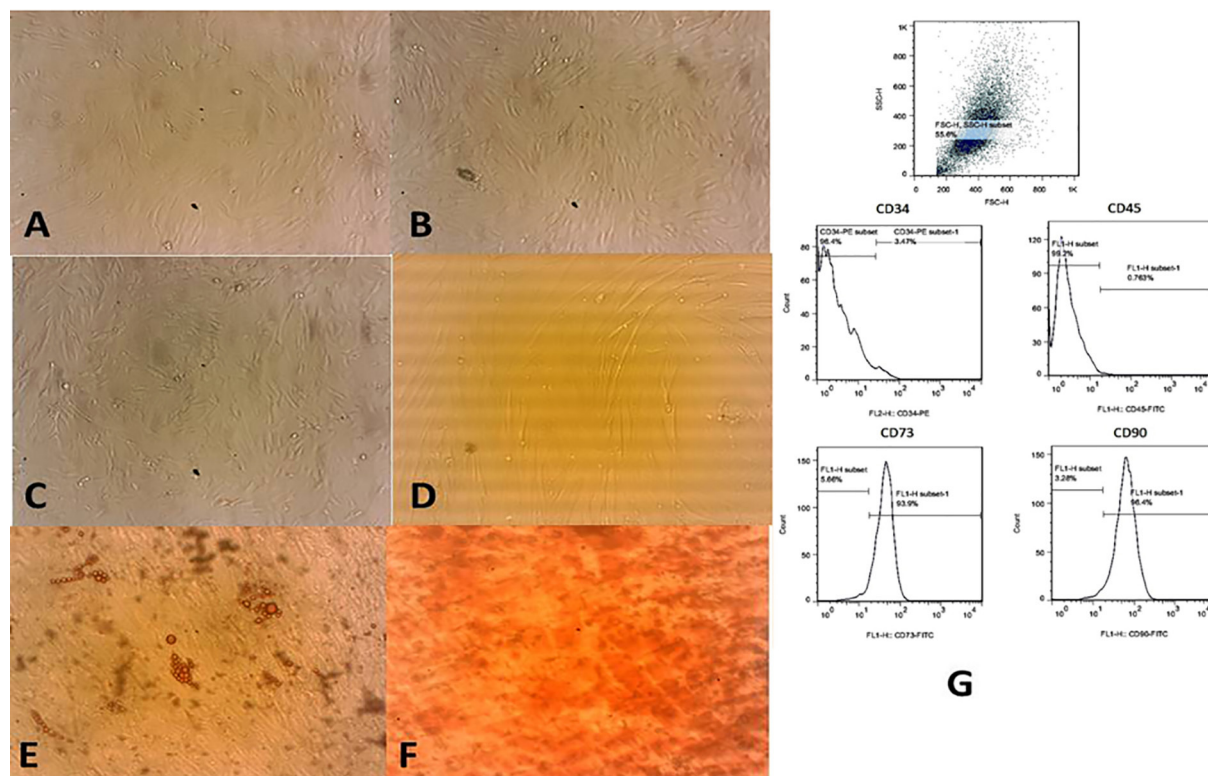


Figure 1: Stem cell characterization was performed to assess mesenchymal properties. Characterizing the AdSCs by morphology (A: Passage 1, B: Passage 2, C: Passage 3), D: Passage 4; Differentiation (E: Adipogenic induction using Oil Red O staining, F: Osteogenic induction using Alizarin Red staining,) and G: flow-cytometry. AdSCs: Adipose tissue-derived stem cells

Regarding adipogenic differentiation property, AdSCs that were stained with Oil Red O illustrated intracellular lipid droplets in red color (figure 1E), confirming the mesenchymal property of the cells. In osteogenic differentiation of the AdSCs, calcium deposits were visible in red color using Alizarin Red staining, which denoted to mesenchymal property of isolated cells (figure 1F). Finally, in characterization of AdSCs for mesenchymal property, the cells were depicted to be positive for expression of CD73 and CD90 and negative for expression of CD34 and CD45 (figure 1G).

MTT Assay

MTT assay denoted to a decrease in cell viability at doses greater than 10 μM of lithium chloride, while the least number of cells was visible at dose of 200 μM of lithium chloride. In this test, different concentrations of lithium chloride were compared with 0% lithium chloride. Based on statistical results, the optimum dose for the highest viability was found to be 6 μM . Because a significant increase was observed at

a dose of 6 μM (shown in green on the graph) and 0 μM . ($P=0.006$, figure 2).

Growth Kinetic

Figure 3 demonstrated the growth curve of AdSCs treated with 6 μM of lithium chloride in comparison to the control for 6 following days that reveals an increase in proliferation of the AdSCs that was not statistically significant ($P=0.067$).

qPCR Assessment

The qPCR illustrated that BAX mRNA pro-apoptotic gene expression in cells treated with 6 μM of lithium chloride had a significant decreasing effect when compared with the control cells not exposed to lithium chloride and the difference was statistically significant ($P=0.028$, figure 4). The *Bcl-2* anti-apoptotic gene in cells treated with 6 μM of lithium chloride showed an increasing impact when compared to the control cells not treated with lithium chloride, and the difference was not statistically significant ($P=0.071$, figure 4).

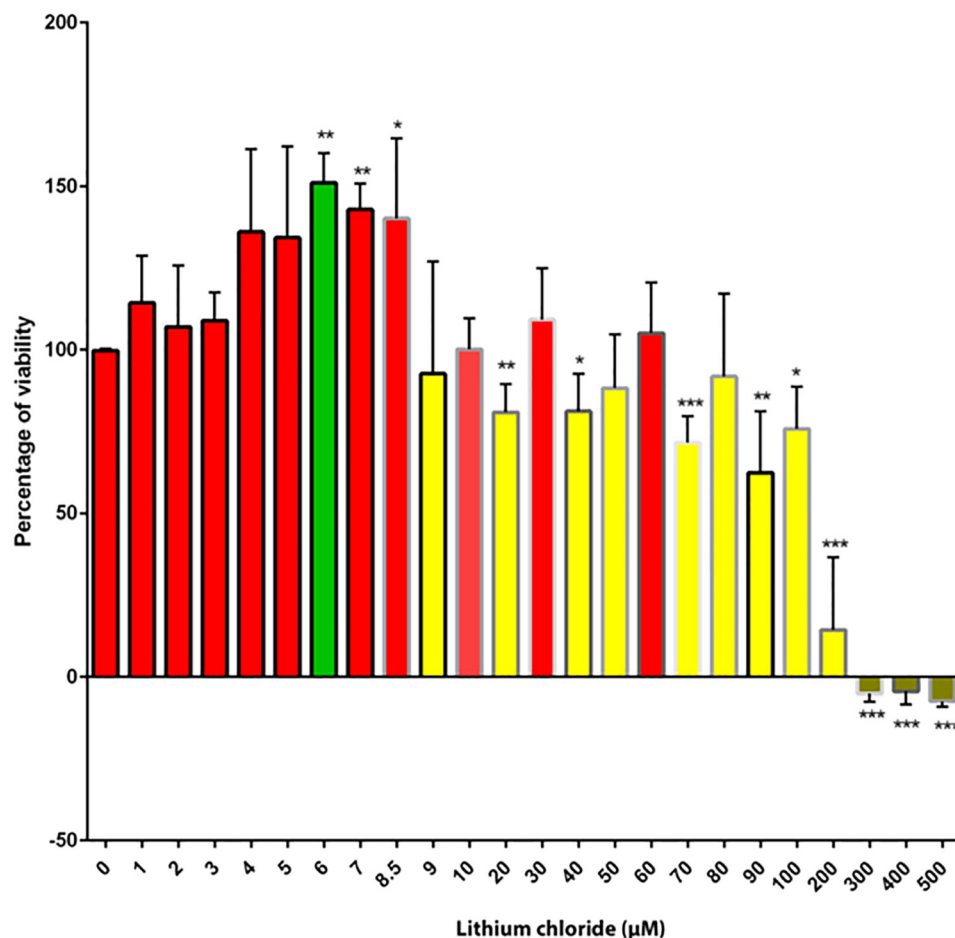


Figure 2: A comparison of the mean \pm SD of cell viability percentages was performed among the different doses of lithium chloride. MTT assay for different doses of lithium chloride (from 10 μM to 200 μM) subjected to AdSCs was illustrated, while the optimum dose for the highest proliferation was found to be 6 μM with a significant difference (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). Data were analyzed by one-way ANOVA with Tukey's *post hoc* test. AdSCs: Adipose tissue-derived stem cells

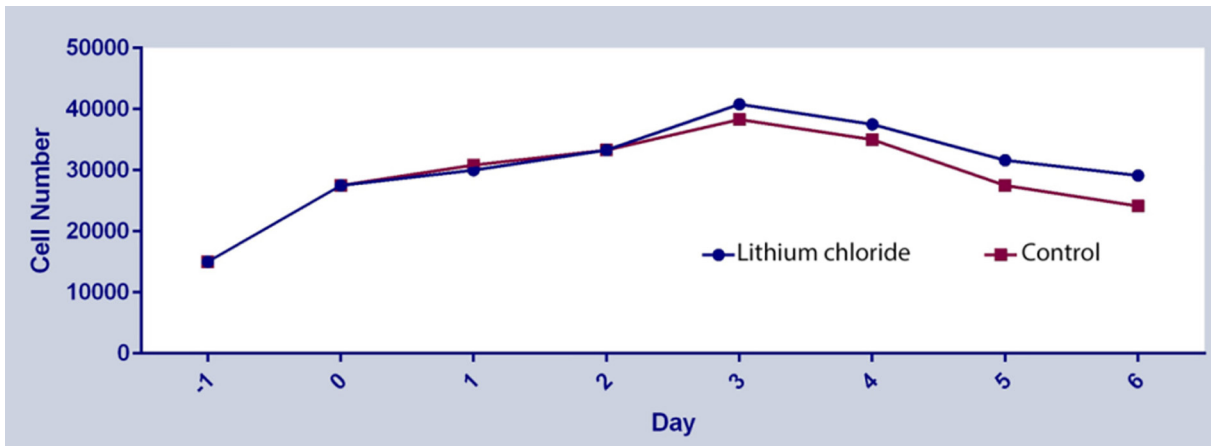


Figure 3: Growth curve of AdSCs treated with 6 μ M of lithium chloride was displayed in comparison to the control for 6 following days ($P=0.067$). A repeated-measures ANOVA assessed the data, with time as the repeated variable. AdSCs: Adipose tissue-derived stem cells

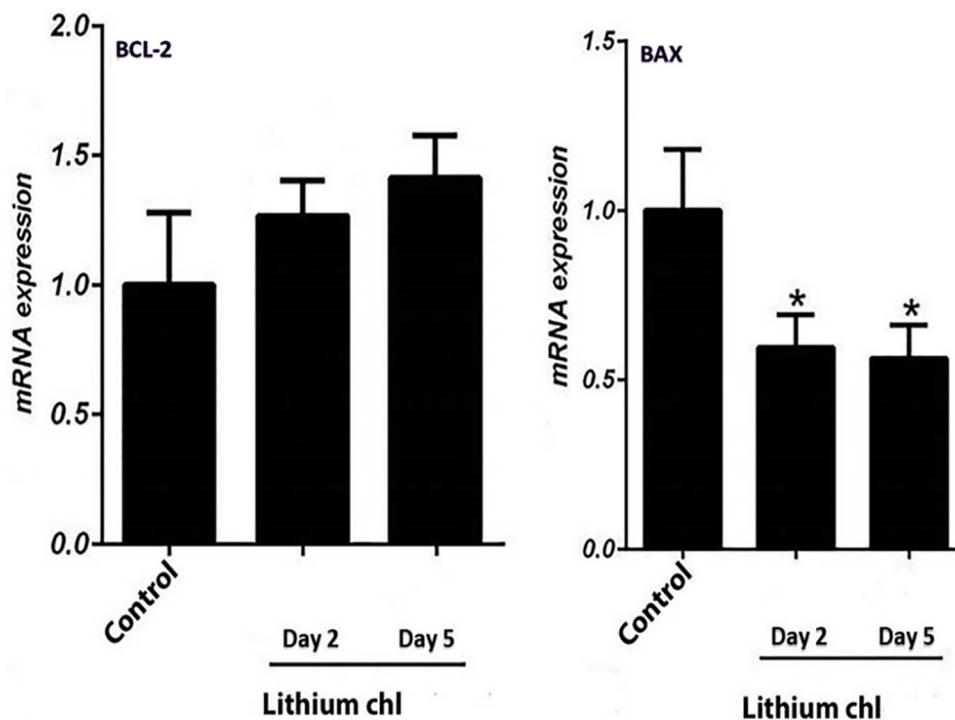


Figure 4: A comparison of the mean \pm SD of mRNA expression levels of Bcl2 and BAX was performed. The effect of lithium chloride on the expression of pro-apoptotic genes (*Bcl-2* and *BAX*) was exhibited in AdSCs treated with 6 μ M of lithium chloride, which was evaluated by real time-polymerase chain reaction (PCR). The results showed that despite the increase in Bcl-2 expression on days 2 and 5, this difference was not significant. However, a significant difference was seen between the treatment and control groups on days 2 and 5 ($*P<0.05$). A repeated-measures ANOVA assessed the data, with time as the repeated variable. AdSCs: Adipose tissue-derived stem cells; Lithium chl: Lithium chloride

Discussion

The present study demonstrated that lithium chloride exerted dosedependent effects on AdSCs. MTT assay revealed a decline in cell viability at concentrations above 10 μ M, with the lowest viability observed at 200 μ M, whereas the optimum dose for maintaining the highest viability was identified as 6 μ M. At the molecular level, qPCR analysis indicated a significant reduction in the proapoptotic gene *BAX* ($P=0.028$), while the antiapoptotic gene

Bcl2 exhibited a nonsignificant increase in expression.

AdSCs are widely used and are powerful sources of cell-based transplantation therapies.^{6, 7, 23} There is always a need to apply safe and simple methods to increase the cell number and prevent cell apoptosis in serum deprivation conditions in the absence of growth factors and genetic engineering techniques.^{1, 24} Autophagy activation has been mentioned as a novel mechanism to protect MSCs from hypoxia and serum deprivation via the AMP-activated

protein kinase of mammalian.²⁵ Lithium treatment can be one of the simple methods in this regard that can be safely added to cell cultures²⁶ and has been approved by the food and drug administration (FDA) for bipolar psychiatric disorders.²⁷ It acts by inhibiting the GSK3 β pathway, the phosphoinositide signaling pathway, and adenylate cyclase, while activating the Wnt/ β catenin signaling pathway.²⁸

In our study, the addition of lithium chloride to cell culture media did not induce any abnormality in cell morphology and increased cell proliferation. Zhang and others similarly showed that lithium chloride could promote the proliferation abilities of neural stem cells *in vitro*, which was potentially mediated by activation of the Wnt signaling pathway.²⁹ Based on these findings and identical to our results, these authors in another study reported that lithium may have therapeutic potential in cell transplantation strategies.³⁰ *In vivo*, the ameliorating effect of lithium chloride was also demonstrated when it was added to human neural stem cells (NSCs) in spinal cord injuries of a rat model.³¹

Kazemi and colleagues illustrated that lithium chloride decreases apoptosis and suggested that autophagy induction may be responsible for this decline.²⁶ Our study identically revealed the protective effect of lithium chloride from apoptosis when it was added to culture medium of AdSCs. de Cates and colleagues similarly found that lithium chloride can increase proliferation of bone marrow-derived mesenchymal stem cells (BMSCs) without any abnormality in cell phenotype and differentiation, and displayed the protective impact of lithium chloride from apoptosis in BMSCs under serum deprivation conditions.³² The same results were noted in our study when AdSCs were subjected to lithium chloride by increasing the expression of *Bcl-2* and reducing the expression of proapoptotic *BAX* gene as described before.³³ Yu and others revealed that lithium chloride did not induce any negative effect on osteogenic/adipogenic differentiation of BMSCs via activation of the β -catenin pathway.³⁴ In contrast, Zanni and colleagues displayed that lithium does not protect from apoptosis and suggested that it may be due to accumulated DNA damage.³⁵

Several studies revealed that lithium can function in numerous molecular pathways, such as the Wnt signaling pathway, glycogen synthase kinase 3 (GSK-3), brain-derived neurotrophic factor (BDNF), the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway, glutamate receptors, and mTOR. Via these pathways, lithium was demonstrated to

impact apoptosis, autophagy, inflammation, excitotoxicity, ferroptosis, and many other pathological processes to improve the CNS damage caused by various etiologies.¹⁶ Animal studies have also illustrated that lithium chloride has the potential to alleviate inflammatory diseases by modulating immune responses, a reduction in pro-inflammatory cytokines, and enhancing anti-inflammatory responses by a decrease in interleukin-2 (IL-2), IL-6, IL-10, and interferon gamma (IFN- γ) secretion.³⁶ It also inhibits neuronal loss and decreases neuroinflammatory markers in bipolar disease patients. It can promote cell survival in these patients, which is in agreement with our *in vitro* findings.^{10, 27, 36}

It seems lithium chloride can have clinical applications as it was demonstrated that it can significantly improve bone regeneration and mineralization during healing of bone defects via activating the Wnt/ β -catenin pathway, promoting M2 macrophage polarization, and enhancing stem cell proliferation and differentiation.³⁷ It was also shown that lithium chloride can prevent glucocorticoid-induced osteonecrosis of femoral heads and increase mesenchymal stem cell proliferation and osteogenic differentiation.³⁸ It should be mentioned that anti-apoptosis activity of lithium chloride can enhance its clinical application as well. Ramushu and colleagues exhibited that lithium chloride can induce oxidative stress, apoptotic cell death, and G2/M phase cell cycle arrest in cancer cells in addition to immunomodulatory effects.³⁹ It was suggested that lithium chloride can efficiently enhance mitomycin C-induced apoptosis and *BAX* and *Bcl-2* expression that can provide a clinical therapeutic strategy for lithium chloride.⁴⁰

A limitation of this study was the lack of detailed mechanistic evidence supporting the observed effects. While our data that lithium chloride enhanced proliferation and reduced apoptosis in AdSCs, the precise signaling pathways involved were not directly examined. Future work should investigate molecular mechanisms such as GSK3 β inhibition, Wnt/ β catenin activation, or mTOR signaling to confirm the basis of these findings.

Conclusion

Our findings denote to the point that addition of lithium chloride to the culture media can increase the proliferation of AdSCs and protect the cells from apoptosis. These findings can be added to the literature when faster cell proliferation and the absence of apoptosis in regenerative and personalized medicine are targeted.

Acknowledgment

This study was extracted from a PhD thesis by Mahin Homayoon (A.143/2017.03.20). We appreciate Ghadir Mother and Child Hospital for sample preparation and Mr. Shahrokh Zare and Dr Iman Jamhiri at Stem Cell Technology Research Center of Shiraz University of Medical Sciences for laboratory collaboration.

Authors' Contribution

M.H: Laboratory works and reviewing the manuscript; D.M: Study design, laboratory works and drafting; M.A.E: Study design, data analysis, and reviewing the manuscript; M.S: Study design and reviewing the manuscript; M.A: Adipose tissue sampling and reviewing the manuscript. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the study to ensure that questions related to the accuracy or integrity of any part of the study were properly investigated and resolved.

Declaration of AI

The authors declare that no AI tools were used in the preparation of this manuscript.

Conflict of Interest: None declared.

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